SUPPRESSION OF DIFFERENT PHASES OF SYSTEMIC CONTACT HYPERSENSITIVITY BY UROCANIC ACID OXIDATION PRODUCTS.

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Abbreviations: CHS, contact hypersensitivity; DTH, delayed type hypersensitivity; ImAc(.Na), imidazole-4-acetic acid as free acid or as sodium salt; ImCHO, imidazole-4-carboxaldehyde; ImCOO(H)(.NH_4)(.Na), imidazole-4-carboxylic acid as free acid or as salt; PCl, picryl chloride (= TNCB, trinitrochlorobenzene); PBS, phosphate buffered saline; PO-mix, mixture of UOPs; ROS, reactive oxygen species; Triplet-mix, mixture of ImCHO, ImCOOH and ImAc 1:1:1; UCA, urocanic acid; UOPs, urocanic acid oxidation products.

ABSTRACT

Upon exposure to UVB, the epidermal component *trans*-urocanic acid is not only photoisomerized into *cis*-urocanic acid, but will also, at least in part, be photooxidized into urocanic acid oxidation products. We hypothesized that urocanic acid oxidation products can mimic UV-induced systemic immunosuppression comparable to the suppressive properties already established for *cis*-urocanic acid. A crude mixture of urocanic acid oxidation products showed a significant suppression of the sensitization phase of the systemic contact hypersensitivity response to picryl chloride. Three of the urocanic acid oxidation products were selected for this study: imidazole-4-carboxylic acid, imidazole-4-carboxaldehyde and imidazole-4-acetic acid. Effects on the sensitization-, elicitation- and post-elicitation phase of contact hypersensitivity to picryl chloride in BALB/c mice were studied and compared to the effects of *cis*-urocanic acid. Imidazole-4-carboxaldehyde was equally effective at suppressing the sensitization phase as *cis*-urocanic acid. The triplet combination of the imidazoles showed more pronounced suppression than that induced by *cis*-urocanic acid. The most effective compounds for the suppression of the elicitation phase appeared to be imidazole-4-acetic acid and *cis*-urocanic acid. Significant suppression of the post-elicitation phase was only obtained with the triplet combination of imidazole-4-carboxaldehyde, imidazole-4-carboxylic acid and imidazole-4-acetic acid, which combination appeared to be effective at all three tested phases. Because these three urocanic acid oxidation products are present in UVB-exposed human stratum corneum, these compounds may play a role in UVB-induced immunosuppression.

INTRODUCTION

During the last two decades extensive research was carried out to characterize the immunomodulatory effect of urocanic acid (UCA), in particular that of the *cis*-isomer, in relation to UV-induced immunosuppression. *Cis*-UCA is formed upon photoisomerization of *trans*-UCA by UV-irradiation of the skin up to a wavelength of approximately 360 nm (1). It was shown that *cis*-UCA can mimic several local as well as systemic immunosuppressive effects of UV on the immune system (2,3). Supporting evidence for *trans*-UCA, as the photoreceptor, and *cis*-UCA, as the immunosuppressant, was obtained from mouse models for delayed type hypersensitivity (DTH) and contact hypersensitivity (CHS)(2,3). Although *cis*-UCA shows clear immunosuppression *in vivo*, no inhibitory effect of *cis*-UCA could be demonstrated in various assays *in vitro* (4-7). Therefore, the mechanism of *cis*-UCA remains still to be resolved.

There is strong evidence that the epidermis undergoes a relatively high level of oxidative stress during UV-irradiation leading to the formation of reactive oxygen species (ROS) and other free radicals (8-13). UCA itself can take part in several radical reactions and can be responsible for the UV-A induced generation of singlet oxygen and hydroperoxides, at least *in vitro* (11-13). Through Fenton-chemistry or UV-irradiation ($\lambda < 320$ nm) peroxides can yield hydroxyl radicals (8-10). *Trans*-UCA and UV-induced *cis*-UCA are present at relatively high concentrations in the epidermis and may form potential targets for hydroxyl radicals. It was previously shown that urocanic acid isomers are efficient hydroxyl radical scavengers (14) and several oxidation products are formed during scavenging *in vitro* as well as in UVB-exposed human stratum corneum (15).

We suggested that - in addition to *cis*-UCA - UCA oxidation products (UOPs) might be involved in the process leading to UV-induced immunosuppression. This idea was raised by the following observations. First, similar levels of *cis*-UCA can be induced by UVA and UVB,

whereas UVB, but not UVA, is a principal inducer of immunosuppression (16). Second, UVB, and not UVA, causes the fission of hydrogen peroxide into hydroxyl radicals and the subsequent formation of the UOPs, studied here (15). Third, antioxidants counteract on UV-induced and *cis*-UCA induced immunosuppression (17,18). Apparently, prevention of the oxidation of UCA into these UOPs might be associated with the abrogation of the suppressive mechanism.

To demonstrate a possible role for UOPs in the phenomenon of UV-induced immunosuppression, the suppressive capacity of three defined UOPs was tested in a mouse model of CHS and compared with the well-known effects of *cis*-UCA. The sensitization -, elicitation - and post-elicitation phase of a systemic CHS-model were studied, using picryl chloride (identical to TNCB) as an hapten. Immunosuppressive effects of the test compounds were monitored as systemic effects, except for the (post-)elicitation phase, in which observed effects were local. The suppression of the post-elicitation phase represented a new procedure and was used to mimic the situation of topical drug application in men after an outbreak of allergic contact dermatitis.

MATERIALS AND METHODS

Materials. Imidazole-4-carboxylic acid (ImCOOH), imidazole-4-carboxaldehyde (ImCHO), imidazole-4-acetic acid (sodium salt; ImAc.Na) were supplied by Sigma-Aldrich/Fluka Chemie BV (Zwijndrecht, The Netherlands). Imidazole-4-carboxylic acid was also used as ammonium - or sodium salt by neutralizing the acid with either concentrated ammonia or sodium hydroxide, followed by vacuum drying. *Cis*-UCA was kindly offered by dr. W.M.P.B. Menge (Free University, Amsterdam, The Netherlands). Picryl chloride (PCl; Chemotronix, Swannanoa, NC, USA) was used as contact sensitizer. The monoclonal antibody against *cis*-UCA was kindly supplied by dr. M. Norval (University of Edinburgh, Scotland). The test compounds for subcutaneous and topical applications to the mice were not purified any further (purity > 98%).

Mice. Male BALB/c mice (8-10 weeks of age) were from the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). The animals (BALB/c/Rivm) were kept in light-, humidity- and temperature-controlled rooms in the animal facility, already two weeks prior to the experiment. They were fed *ad libitum* with water and Hope Farm Chow (SRM-A).

Contact Hypersensitivity (CHS). PCl was recrystallized three times from methanol/water before use and was protected from light and humidity during storage at 4^0 C. For active contact sensitization, mice were sensitized with PCl on day 1 by topical application of 150 μL of 5 % PCl in ethanol/acetone (3:1) on the shaved abdomen and thorax. In addition, 25 μL was applied on each foot pad. On day 5, mice were challenged on both sides of each ear by topical application of one drop of 0.8 % PCl in olive oil. Duplicate measurements of ear thickness were made before elicitation and at 24 h after ear challenge with an engineer's micrometer (Mitutoyo model 193-10, Tokyo, Japan). Nonsensitized (negative) control

animals were only PCl-challenged and measured in the same way. These background ear-swelling responses were subtracted from the responses in sensitized mice to finally obtain the net ear-swelling. Ear thickness was expressed as relative values in percent (%) of positive control values from sensitized, placebo-treated mice. The mean ± SEM of ear thickness measurements is presented in the figures. In one experiment, a monoclonal anti-*cis*-UCA antibody solution (1 x 100 μL) was given intraperitoneally 2 h before the test compounds were administered.

Suppression of the sensitization phase of CHS. One hour prior to the sensitization with PCl, as outlined above, mice were subcutaneously administered two doses of 0.1 mL of test compound (1 g/L) in PBS in the neck area (100μg ~ 1 μmol imidazolic UOP) . To compare, the UOP-concentrations found in UV-irradiated human stratum corneum amounted 7, 140 and 4 μmol per 100 gram for ImCHO, ImCOOH and ImAc, respectively (15). Positive and negative control mice were administered PBS only.

Suppression of the elicitation phase of CHS. Fifty microliters of test compound (10 g/L) in ethanol/water 4:1 were topically applied to the murine ears one day before (on day 4) as well as 20 - 30 min before challenge. The ethanol/water solutions were kept at room temperature in the dark and were argon purged. In this way, the test solutions, and in particular imidazole-4-carboxaldehyde solution, was prevented from oxidative breakdown.

Suppression of the post-elicitation phase of CHS. Twenty-four h after challenge, on day 6, ear swellings were measured. On the same day topical applications (ethanol/water test solutions as described above) on the murine ears were performed at 10 AM and 4 PM, respectively. These two topical applications per day were repeated on day 7, 8 and 9. Additional ear swelling measurements were carried out on day 8, 9 and 10 before the first topical application

of that day. The time-frames of the measurements are referred to as 24, 72, 96 and 120 h post-elicitation.

The UCA photooxidation mixture (PO-mix). The mixture of photooxidized products, derived from UCA, is referred to as PO-mix (15). *Cis*-UCA was formed in the mixture as well, but due to excessive break down of UCA into photooxidation products, the PO-mix contained less than 3 % of each UCA isomer. The PO-mix, a light-yellow product, is readily soluble in water media and contains, among others, the three imidazoles.

Statistical analysis. Levels of significance were calculated using Welch's unpaired t-test. One sided p-values < 0.05 and < 0.01 were considered as significant and highly significant, respectively. "n" represents the number of mice for each experiment.

RESULTS

A crude mix of UOPs (PO-mix) and *cis*-UCA suppress the sensitzation phase of CHS

The PO-mix and *cis*-UCA were tested for their capacity to suppress CHS responses against PCl. The dissolved test compounds were subcutaneously injected one hour prior to sensitization with PCl and were compared with sham-treated, sensitized mice (positive controls). The PO-mix at concentrations of 2 and 0.2 g/L suppressed the CHS response in a strong, highly significant ($p < 0.01$) manner (19 and 29 % ear swelling, respectively). These suppressions were not significantly different from the effect (31 %) of the 'classical' immunosuppressant *cis*-UCA (**Fig. 1**).

Although the overall concentration of the PO-mix (2 g/L) was twice that of *cis*-UCA (1 g/L), it must be noted that the concentrations of effective compounds in that mix must be much lower than that of *cis*-UCA. A tenfold dilution of the PO-mix (0.2 g/L) was still as effective as *cis*-UCA.

Pure, defined imidazolic UOPs suppress the sensitization phase of CHS as well

The three imidazoles that were identified (15) within the PO-mix (i.e. ImCOOH, ImCHO and ImAc) were separately tested for their capacity to suppress the CHS response at a concentration of 1.0 g/L. Only ImCHO showed highly significant ($p < 0.01$) reduction, whereas ImCOOH and ImAc showed significant ($p < 0.05$) reductions of the CHS response, similar to the effect of *cis*-UCA (**Fig. 2**). Another identified UOP, glyoxylic acid, and its proposed oxidation product, oxalic acid (15), were also tested as neutral ammonium salts for their effects on the CHS response, but they did not show any significant suppressive effect (data not shown).

In order to further investigate the immunosuppressive effects of the imidazoles, a series of dose-suppression studies was performed. To that end, the imidazoles and *cis*-UCA

were tested in a separate experiment at 1.0 g/L and at 3 subsequent steps of 5-fold dilutions in PBS. The imidazoles showed good inverse correlations between concentrations and percentages of relative ear swellings, determined by linear regression analysis ($R = 0.975$ - 0.989). For *cis*-UCA less correlation was observed ($R = 0.87$). These results proved that a dose-suppression relation exists and indicated that imidazole have the ability to reduce the murine ear swelling responses in the sensitization phase of CHS at concentrations as low as 0.04 g/L (1.4 on -log scale). From the position of the slope it can be derived that ImCHO was the most effective compound and ImAc was the least effective compound **(Fig. 3)**.

For the situation *in vivo*, the three imidazoles, ImCOOH, ImCHO and ImAc (here referred to as A,B and C, respectively) are concurrently generated in the skin upon oxidative stress (15). Therefore, the three imidazoles were tested in several combinations to investigate possible synergistic effects on the suppression of the CHS response. In doublet (AB, BC, AC) or triplet (ABC) combinations each imidazole was kept at a concentration of 0.33 g/L. In addition, the triplet combination was also tested at a 4 times lower concentration (0.08 g/L for each imidazole). The application of the AC and AB combinations resulted in a significant suppression ($p < 0.05$) compared to positive control. The other combinations and *cis*-UCA resulted in very significant ($p < 0.01$) suppressions compared to positive control. Moreover, the application of both ABC combinations of 1.0 and 0.25 g/L, but not the BC combination, resulted in a significantly stronger ($p < 0.05$) suppression than that of combination AC. Remarkably, a similar suppression of CHS response (24 %) was obtained with this ABC combination at a four times lower concentration **(Fig. 4)**. To summarize, the sensitization phase of the CHS response is strongly suppressed by combinations of the imidazoles, especially by the ABC and BC combinations. However, a synergistic effect of the combined imidazoles could not be demonstrated.

The UVB-induced immunosuppression can be reduced by a monoclonal anti-*cis*-UCA antibody in a herpes simplex virus-induced DTH-model, but not in an oxazolone-induced CHS model (19). We investigated whether this antibody could affect the *cis*-UCA and UOP-induced immunosuppression in our picryl chloride-induced CHS model. The intraperitoneal administration of that monoclonal antibody before the injections of *cis*-UCA resulted in a significant ($p < 0.05$) reduction of the systemic suppression of the sensitization phase. When the same procedure was carried out with the triplet combination of the imidazoles, no effect of the antibody was observed **(Fig. 5)** and a comparable strong suppression resulted as shown in Figure 4.

Pure, defined imidazolic UCA oxidation products and *cis*-UCA suppress the elicitation phase of CHS

Besides the immunosuppressive effects of the UOPs and *cis*-UCA on the sensitization phase of PCl, the effects on the elicitation phase were tested through administration of the test compounds 24 h and 0.5 h prior to the application of PCl to the ears of the sensitized mice. Using this experimental setup, strong suppressions of the elicitation phase could be demonstrated. The strongest suppressions were seen with the application of *cis*-UCA, the triplet mix of imidazoles and ImAc.Na ($p < 0.01$). $ImCOONH_4$ (ImCOOH used as ammonium salt) exhibited a moderate suppression ($p < 0.05$). Remarkably, ImCHO, one of the stronger UOP-suppressants concerning the sensitization phase, did not show any suppressive effect on the elicitation phase under our test conditions **(Fig. 6)**.

Pure, defined UOPs and *cis*-UCA suppress the post-elicitation phase of CHS

The study design for suppression of the post-elicitation phase was introduced for its relevance for (topical) dermatological therapies. The suppressive effects on the post-elicitation responses are shown in **Fig. 7**. The positive control values were normalized to 100 % at all

time points. Absolute ear swelling values of the vehiculum-treated mice (positive control) showed a decline over the post-elicitation test period of 120 hours (data not shown). In case the test compounds would show an additional reduction over the natural decline in such a period, a so-called suppression of the post-elicitation response would be obtained. At 72 h after elicitation (after 4 topical treatments) all test compounds showed slight reduction of ear swelling, although not significantly different from the 24 h value. At 96 h after elicitation (after 6 topical applications) comparable reductions were observed, except for ImAc.Na. At 120 h after elicitation (after 8 topical applications), *cis*-UCA, ImAc.Na and the triplet mix of imidazoles showed the largest reductions compared to the non-treated 24 h values, although the only significant reduction ($p < 0.05$) was obtained by 8 topical applications of the triplet mix of imidazoles. In these groups of mice, the initial non-treated mean value of 100 % ear swelling was reduced to 66 % after 8 topical applications, while the vehiculum-treated mice (positive control) remained at 100 %. Moreover, the effect of the triplet-mix consisted of an additional gradual decline of swelling across the entire test period, compared to positive control. The 'classical' suppressor of the sensitization phase, *cis*-UCA, did not cause significant suppressive effects (**Fig. 7**).

DISCUSSION

Trans-UCA and *cis*-UCA have been tested in CHS and DTH models many times during the past two decades and predominantly *cis*-UCA was found to exert suppression of CHS and DTH responses (2,3). It should be noted that *cis*-UCA is not unique for the induction of immunosuppressive effects in these CHS and DTH models. Other imidazolic compounds, such as histamine, have previously been shown to suppress TNCB-induced CHS responses and DTH responses to herpes simplex virus (HSV) as well (20,21). The strongest suppressive effects are found with compounds having a substituent at the 4-position of the imidazole ring (e.g. dihydroUCA, histamine and *cis*-UCA). The three imidazolic UOPs, tested in this study, also have 4-substituted imidazole rings. In this respect, the suppressive ability of the three tested imidazoles is in line with this finding. Other 5-membered, non-imidazolic, heterocyclic ring compounds were previously shown (21) to be less able to cause suppression of DTH to HSV, at least in the immunization phase. The use of the monoclonal anti-*cis*-UCA antibody (mAb) showed however a discrimination between the 4-substituted imidazoles, *cis*-UCA and the imidazolic UOPs. The reduction of the immunosuppression (Fig. 5) might be caused by specific binding of *cis*-UCA to the mAb, leading to extraction of *cis*-UCA from the test system. The imidazolic UOPs showed an uninhibited suppression and were apparently not bound by the mAb.

Animal studies on immunomodulators focus on suppression of the sensitization phase of the CHS response. An alteration of the studied phase (e.g. from sensitization to elicitation) can lead to a dramatic shift in the modulatory effect of a given compound as was observed in our study when testing ImCHO in the sensitization phase (Fig. 2) and elicitation phase (Fig. 6). This compound did not render any suppressive effect on the CHS response of already sensitized animals, whereas a substantial suppression was observed when administered prior to sensitization. Either skin penetration with the ethanol/water test solutions may have become

a critical factor or different routes of administration may have caused the modulation of different cellular targets. The vehiculum for topical application contained 80 % ethanol and ethanol is regarded as a penetration enhancer. Because suppressive effects in the elicitation phase are seen, a part of the topically applied UOP compound must have passed the upper skin. The bioavailibilty of UOPs is not yet well defined, although we assume a comparable pharmacological behavior among the studied UOPs because of the similarities in molecular structure.

To summarize the suppressive effects under our test conditions: ImCHO and *cis*-UCA are most effective for suppression of the sensitization phase; ImAc and *cis*-UCA are most effective for suppression of the elicitation phase; the triplet mix of imidazoles is effective for suppression of the post-elicitation phase. Moreover, this mix showed substantial suppression in every phase of systemic CHS response. Basically viewed, a test compound given *prior* to the sensitization phase could have suppressive effects on the elicitation phase as well, especially in case the applied test compound has a long biological half-life. However, our test compounds given *prior* to the sensitization may have affected only that phase, as was assumed in a previous research paper for structurally related *cis*-UCA (22). The three tested imidazoles (and possibly other UOPs) are present in UVB-exposed stratum corneum (15) as a result of enhanced levels of oxidative stress. Related to that, this paper provides an alternative view on a previously established conclusion concerning the role of histamine receptor antagonists / agonists in *cis*-UCA-induced immunosuppression (23). Cimetidine (an H2-receptor antagonist) was tested in the mouse model of DTH response to show that prevention of the proposed *cis*-UCA binding to histamine-like receptors has led to reduction of the *cis*-UCA-induced immunosuppression. Alternatively viewed, cimetidine and the UCA isomers are both good hydroxyl radical scavengers (14,24). Under conditions of oxidative stress with the inherent phenomenon of elevated hydroxyl radical levels, such as during UV-exposure, cimetidine may compete with UCA for hydroxyl radical scavenging. In other words, the

presence of cimetidine may prevent the formation of UOPs from the UCA isomers in UV-irradiated skin. Assuming a predominant role for UOPs in UV-induced immunosuppression, this cimetidine-induced reduction might be due to decreased levels of UOPs. Cimetidine may act in a similar competitive way to yield reduced UOP formation as was proposed for the antioxidants N-acetylcysteine and carnosine (17,18).

The strong reduction of the CHS response, exerted by the PO-mix, is a phenomenon for further investigation. The PO-mix consisted of identified as well as of unidentified UOPs, each of them at concentrations that would be much lower than the commonly used 1.0 g/L, and yet a strong suppression of CHS response resulted (Fig. 1). The three identified imidazoles in that mixture may have contributed to this suppressive effect.

A prominent role for *cis*-UCA in the phenomenon of UV-induced immunosuppression was established more than 20 years ago (25) and frequently confirmed since. An important conclusion from our study is the finding that this suppression is not only accomplished by *cis*-UCA, but by UOPs as well. We have demonstrated that UOPs, which are formed upon UVB-irradiation of the (human) skin and *in vitro*, have an immunosuppressive potential in different phases of CHS, indicating that UOPs play an important role in the process of UV-induced immunosuppression.

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LEGENDS

Figure 1.

Suppression of the sensitization phase of systemic CHS by *cis*-UCA and a crude mixture of UCA photooxidation products (PO-mix). The horizontal axe shows the net (antigen-specific) ear swelling response 24 h after elicitation of the ear pinnea. The net ear swelling of the positive control was 115 μm in this experiment and was set to 100 % . Each bar represents n = 3. The other sample values were converted into percentages by multiplying with the same factor (100/115). The double asterisks refer to a very significant ($p < 0.01$) suppression, compared to positive control.

Figure 2.

Suppression of the sensitization phase of systemic CHS by separate, pure imidazolic UOPs. Each bar represents n = 6, from a duplicate experiment. The averaged net ear swelling of the positive control was 111 μm and was set to 100 %. The other samples were converted by the same factor (100/111). The single and double asterisks refer to a significant ($p < 0.05$) and a very significant ($p < 0.01$) suppression, respectively, when compared to positive control.

Figure 3.

Concentration dependent suppression of the sensitization phase. Linear regression fits were obtained by studying the effects of 4 concentrations of the imidazoles in PBS: 1.00 -, 0.20 -, 0.04 - and 0.008 g/L and were represented by their - log values. The ear swellings were expressed as percentages and each data point represents n = 3.

Figure 4.

Suppression of the sensitization phase of systemic CHS by *cis*-UCA and combinations of the 3 imidazolic UOPs. A = ImCOOH, B = ImCHO, C = ImAc.Na. The net ear swelling of the positive control was 77 μm and was set to 100 %. The other results were converted by the same factor (100/77). The single and double asterisks refer to a significant ($p < 0.05$) and a very significant ($p < 0.01$) suppression, respectively, compared to positive control. The significant ($p < 0.05$) stronger suppression of the ABC combination, compared to the AC combination, has been indicated for both concentrations of ABC. Each bar represents $n = 3$.

Figure 5.

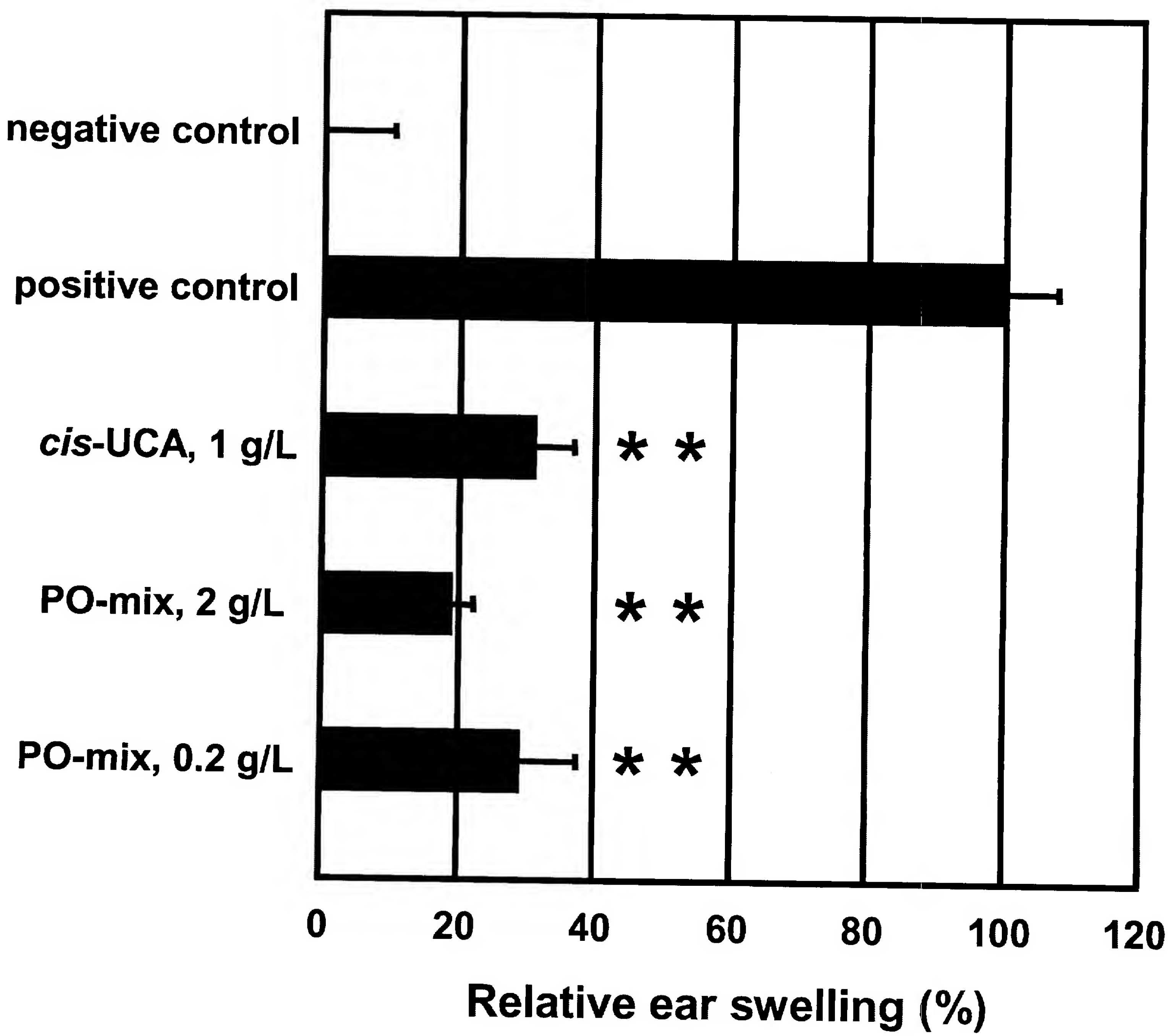
The effect of a monoclonal anti-*cis*-UCA antibody (mAb) on the suppression of the sensitization phase of systemic CHS induced by *cis*-UCA and by the triplet mix of imidazoles. Only with *cis*-UCA administration the mAb showed a significant, opposed effect ($p < 0.05$; single asterisk).

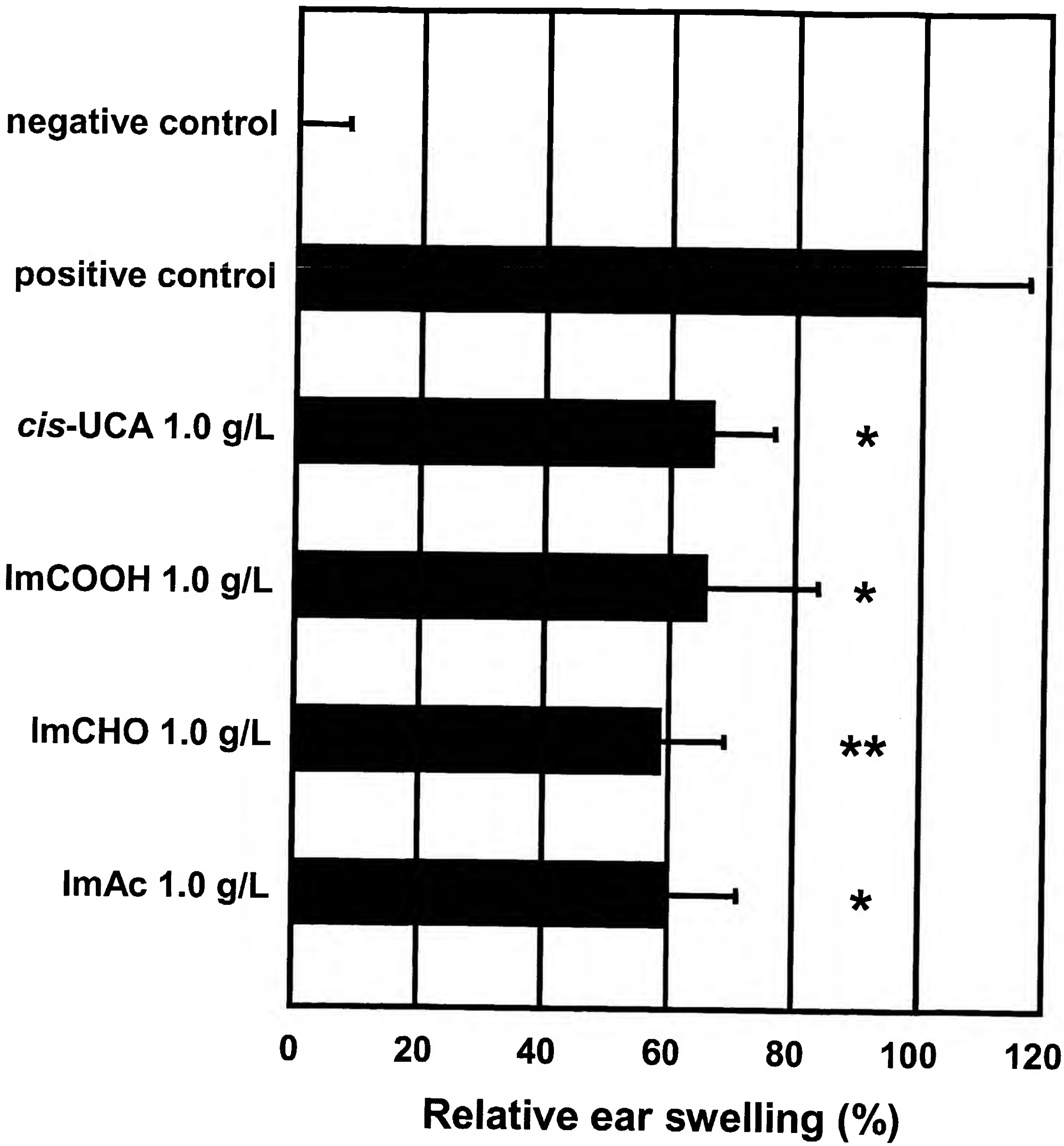
Figure 6.

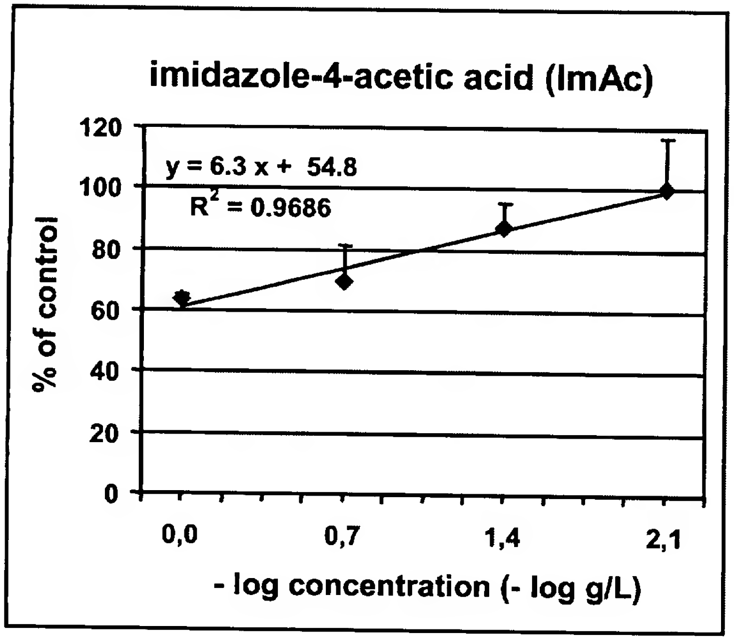
Suppression of the elicitation phase of systemic CHS by *cis*-UCA and the pure imidazolic UOPs. The test compounds were used at a concentration of 10 g/L in ethanol/water 4:1. The triplet mix is a mixture of the three imidazoles, each at 3.3 g/L. The net ear swelling of the positive control was 55 μm and was set to 100 %. The other results were converted by the same factor (100/55). Further procedures are outlined in the Materials & Method section. The single and double asterisks refer to a significant ($p < 0.05$) and a highly significant ($p < 0.01$) suppression, respectively, compared to positive control. Each bar represents $n = 3$, except for positive control and for ImAc.Na: $n = 6$, for *cis*-UCA: $n = 5$.

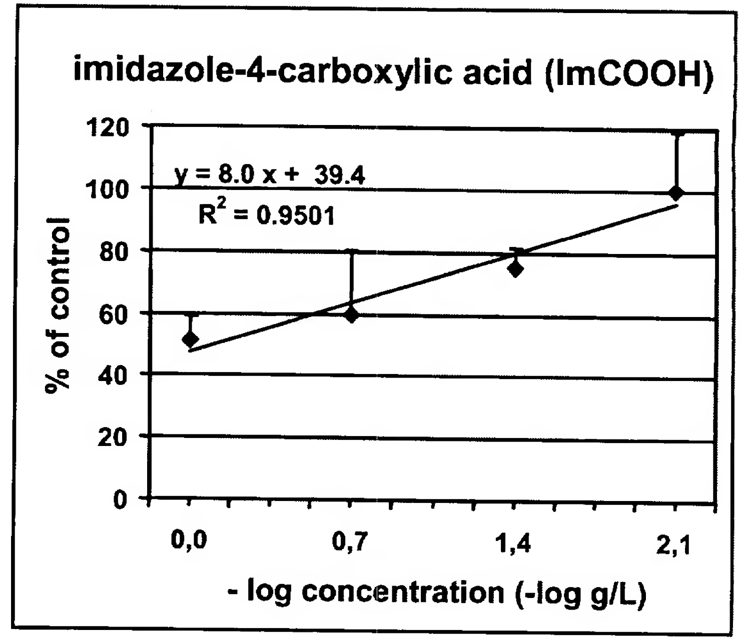
Figure 7.

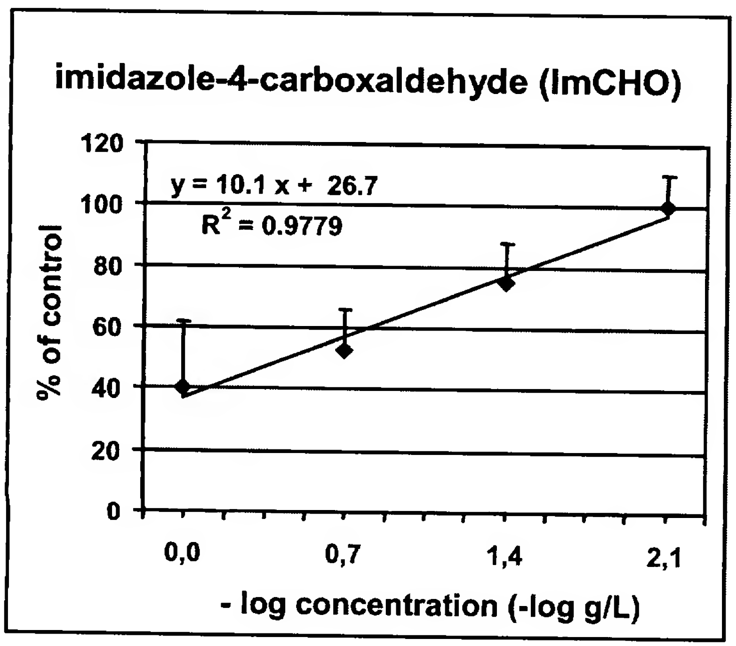
Suppression of the post-elicitation phase of systemic CHS by *cis*-UCA and the pure imidazolic-UOPs. The test compounds were used at a concentration of 10 g/L in ethanol/water 4:1. Relative ear swellings were expressed in percentages and were normalized to the positive control value of the same time-point. The single asterisk refers to a significant ($p < 0.05$) suppression, compared to the 24 h. value. Each bar represents the mean value of three separate experiments (each experiment $n = 3$; total $n = 9$).

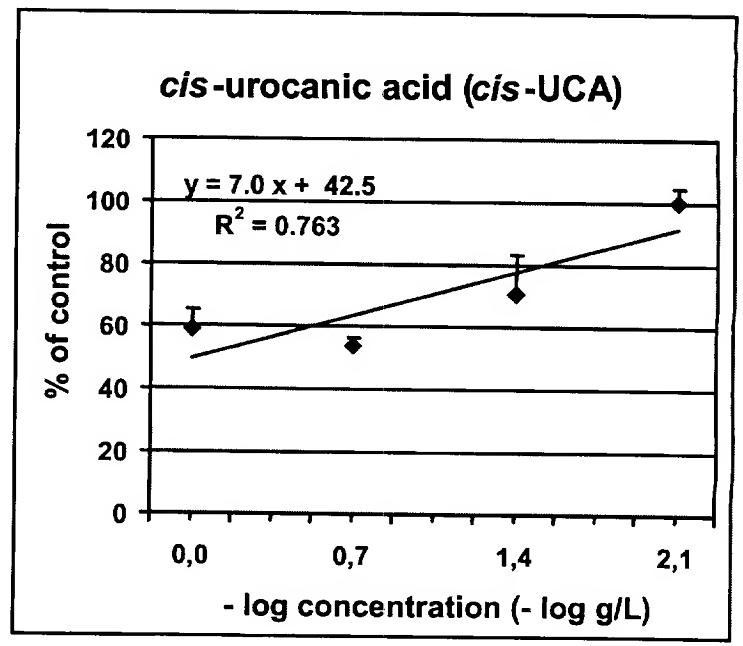

negative control
positive control
cis-UCA, 1 g/L
PO-mix, 2 g/L
PO-mix, 0.2 g/L
* *
* *
* *
0
20
40
60
80
100
120
Relative ear swelling (%)

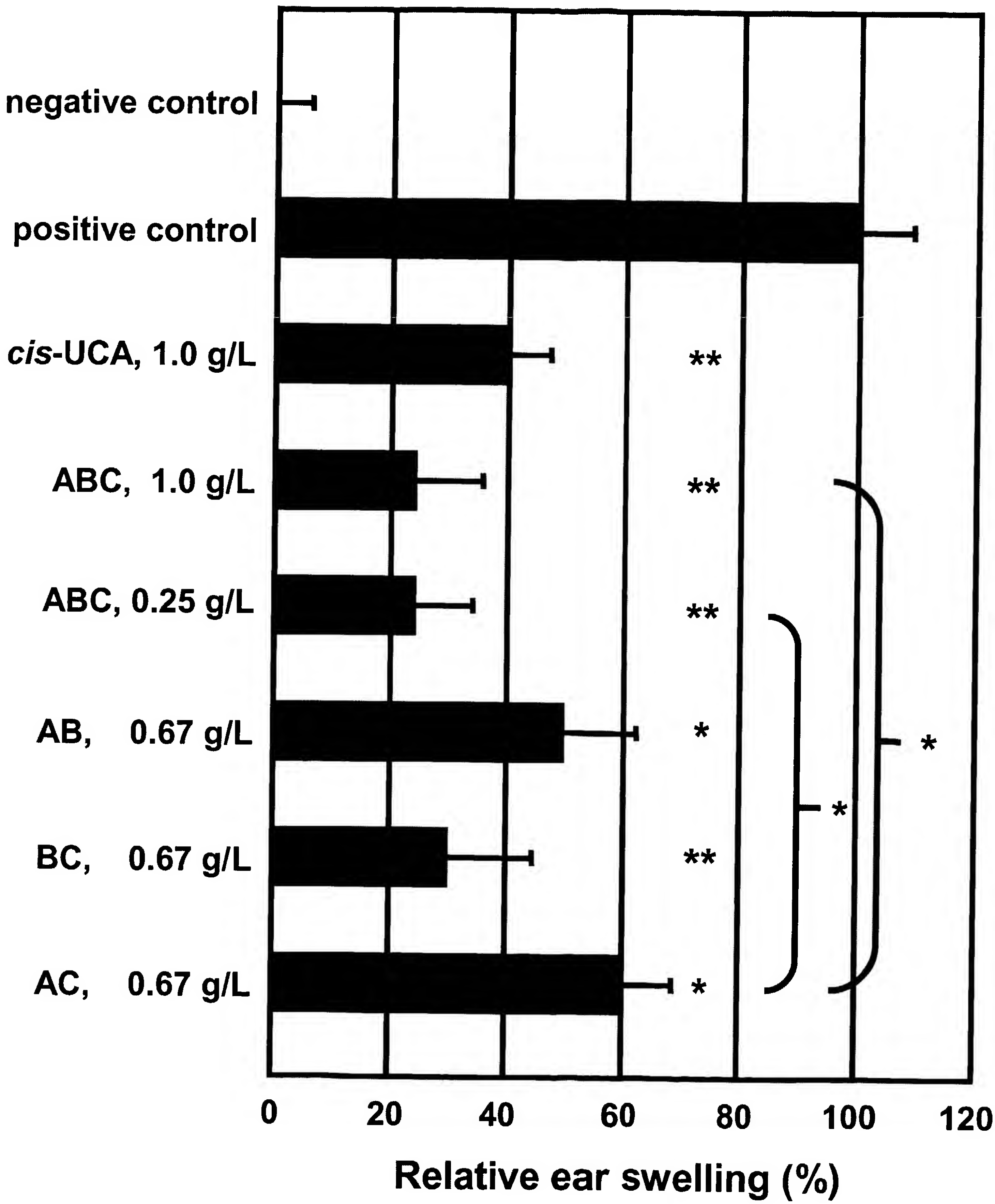

negative control
positive control
cis-UCA 1.0 g/L
ImCOOH 1.0 g/L
ImCHO 1.0 g/L
ImAc 1.0 g/L
*
*
**
*
0
20
40
60
80
100
120
Relative ear swelling (%)

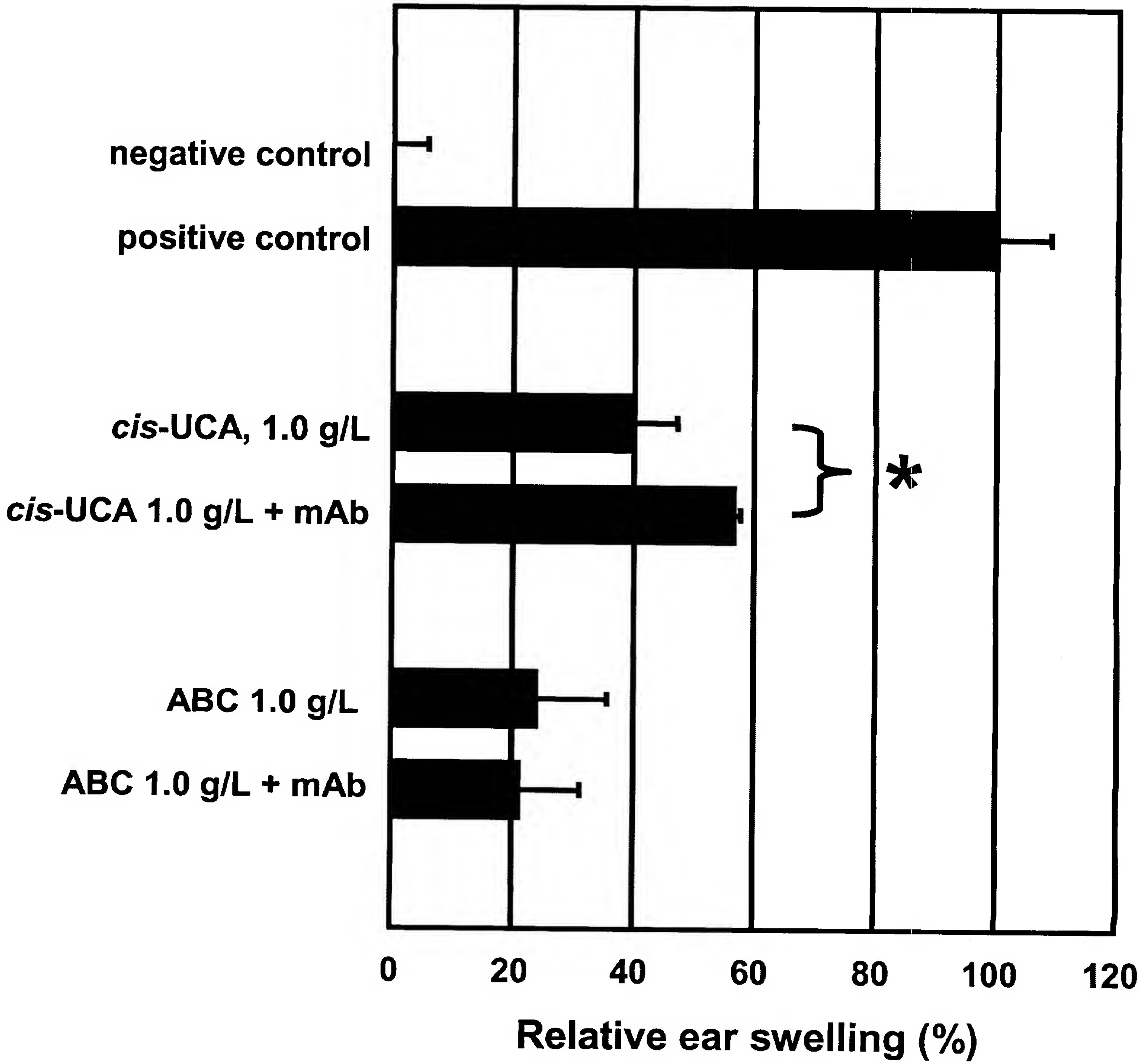

imidazole-4-acetic acid (ImAc)
y = 6.3 x + 54.8
R^2 = 0.9686
% of control
120
100
80
60
40
20
0
0,0
0,7
1,4
2,1
- log concentration (- log g/L)

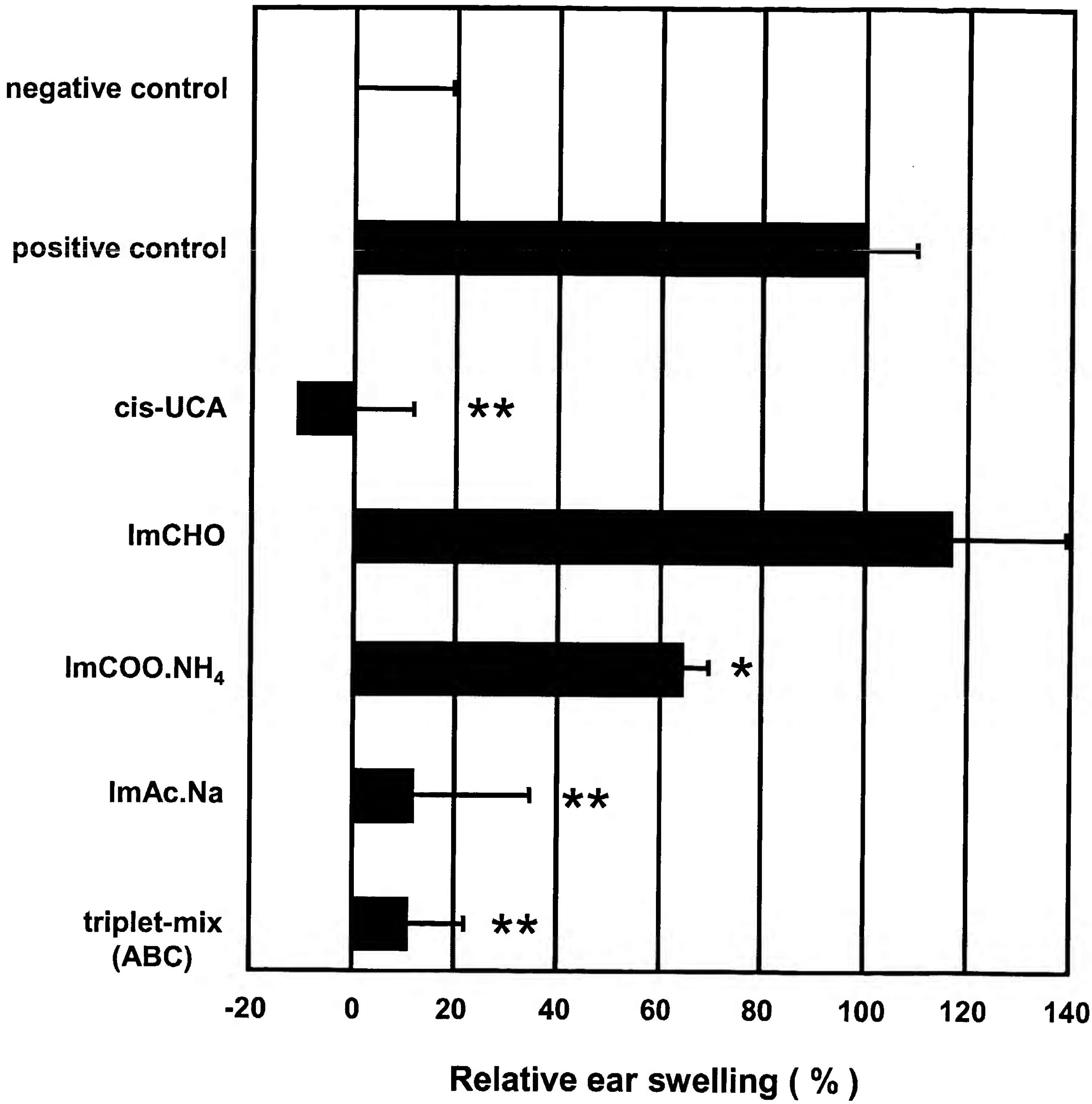

imidazole-4-carboxylic acid (ImCOOH)
y = 8.0 x + 39.4
R^2 = 0.9501
% of control
120
100
80
60
40
20
0
0,0
0,7
1,4
2,1
- log concentration (-log g/L)

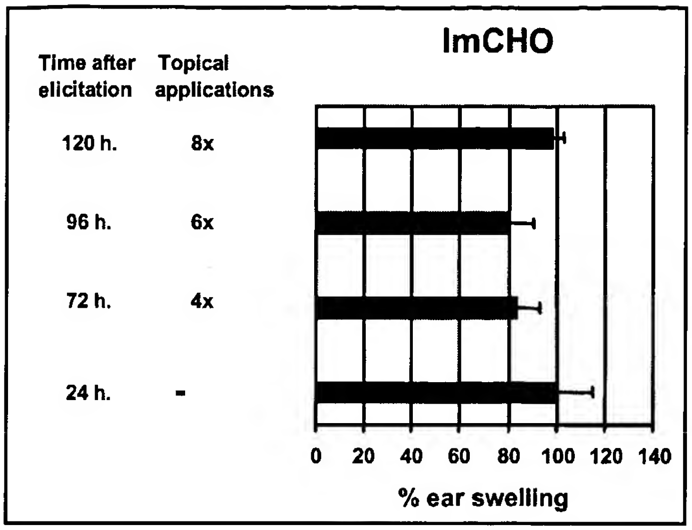

imidazole-4-carboxaldehyde (ImCHO)
y = 10.1 x + 26.7
R^2 = 0.9779
% of control
120
100
80
60
40
20
0
0,0
0,7
1,4
2,1
- log concentration (-log g/L)

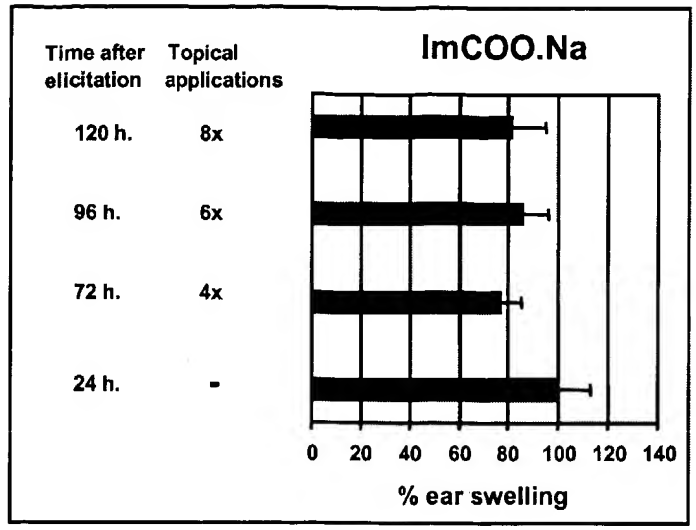

cis-urocanic acid (*cis*-UCA)
y = 7.0 x + 42.5
R^2 = 0.763
% of control
120
100
80
60
40
20
0
0,0
0,7
1,4
2,1
- log concentration (- log g/L)

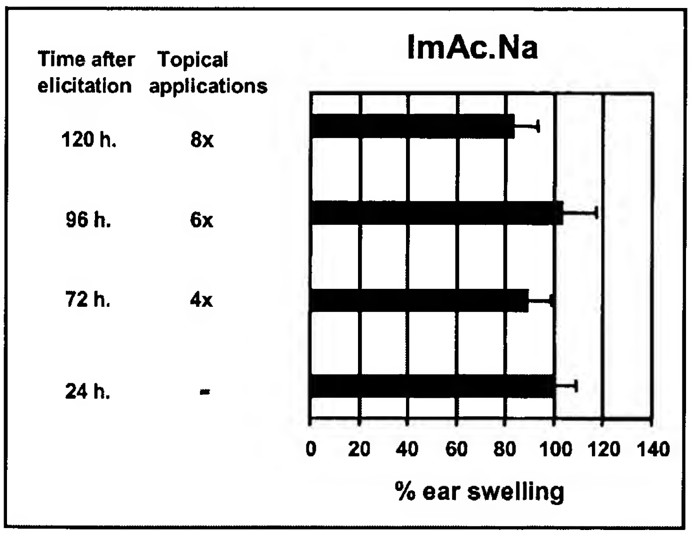

negative control
positive control
cis-UCA, 1.0 g/L
ABC, 1.0 g/L
ABC, 0.25 g/L
AB, 0.67 g/L
BC, 0.67 g/L
AC, 0.67 g/L
**
**
**
*
**
*
*
*
0
20
40
60
80
100
120
Relative ear swelling (%)

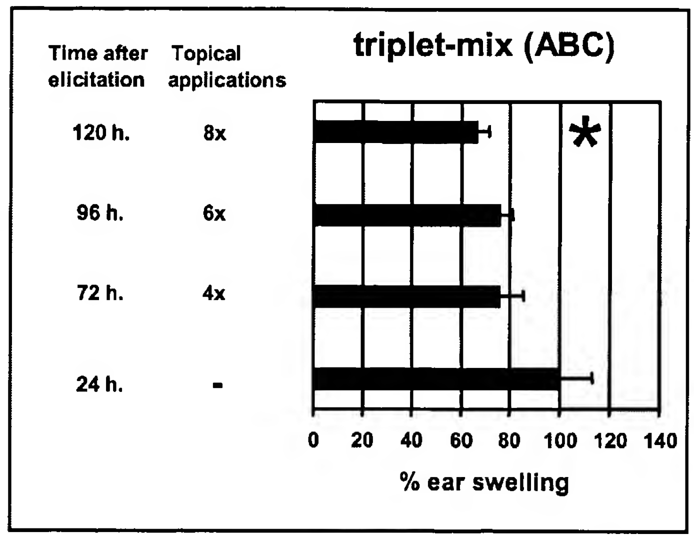

negative control
positive control
cis-UCA, 1.0 g/L
cis-UCA 1.0 g/L + mAb
ABC 1.0 g/L
ABC 1.0 g/L + mAb
*
0
20
40
60
80
100
120
Relative ear swelling (%)

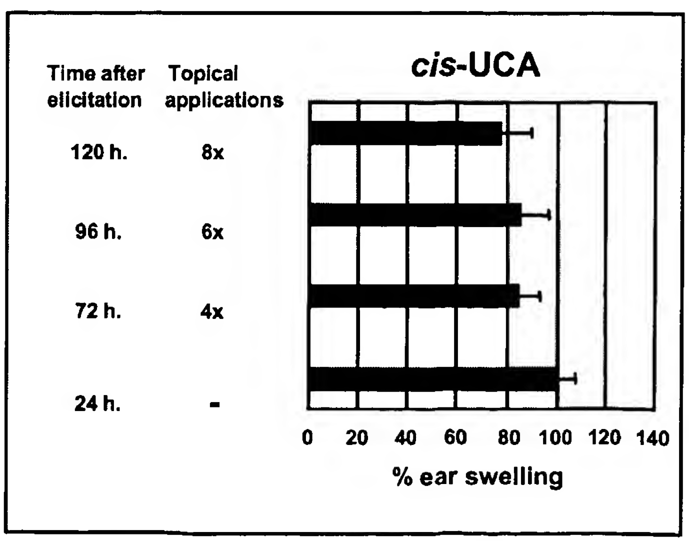

negative control
positive control
cis-UCA
ImCHO
ImCOO.NH4
ImAc.Na
triplet-mix (ABC)
**
*
**
**
-20
0
20
40
60
80
100
120
140
Relative ear swelling (%)


ImCHO
Time after elicitation
Topical applications
120 h. 8x
96 h. 6x
72 h. 4x
24 h. -
0 20 40 60 80 100 120 140
% ear swelling


ImCOO.Na
Time after elicitation
Topical applications
120 h. 8x
96 h. 6x
72 h. 4x
24 h. -
0 20 40 60 80 100 120 140
% ear swelling


ImAc.Na
Time after elicitation
Topical applications
120 h. 8x
96 h. 6x
72 h. 4x
24 h. -
0 20 40 60 80 100 120 140
% ear swelling


triplet-mix (ABC)
Time after elicitation
Topical applications
120 h. 8x
96 h. 6x
72 h. 4x
24 h. -
*
0 20 40 60 80 100 120 140
% ear swelling


cis-UCA
Time after elicitation
Topical applications
120 h. 8x
96 h. 6x
72 h. 4x
24 h. -
0 20 40 60 80 100 120 140
% ear swelling